Parasites and drug resistance

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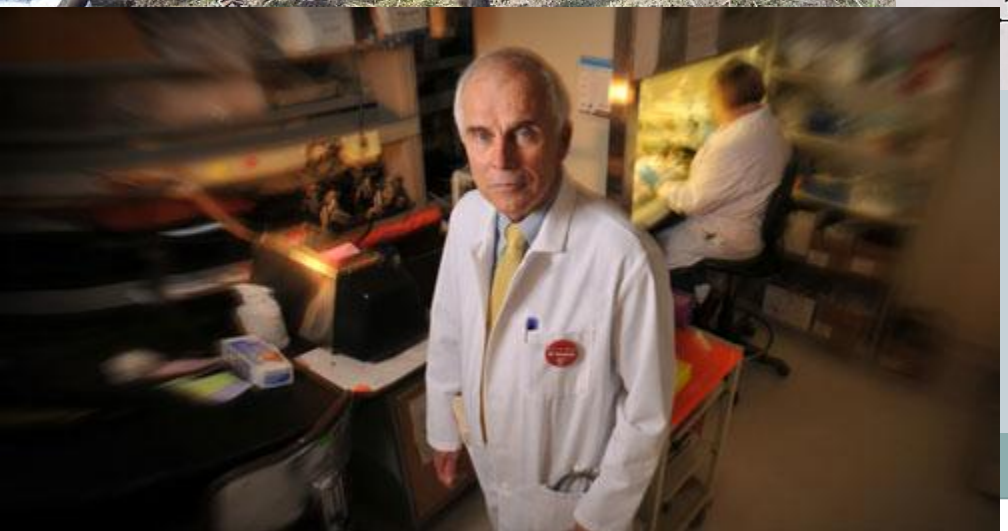
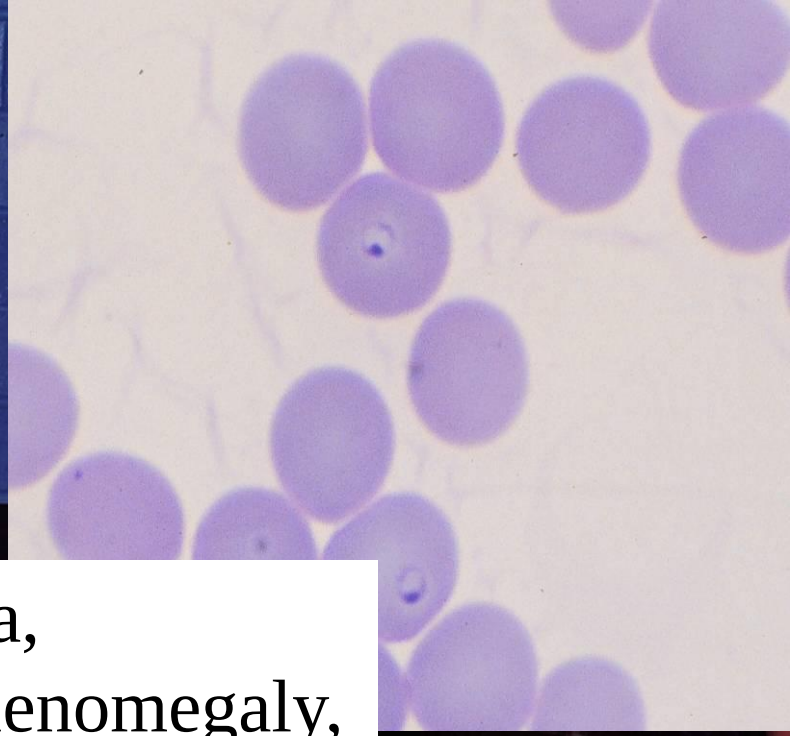
My Life

July 1, 1995:
My first day as a veterinarian



I HATE research…

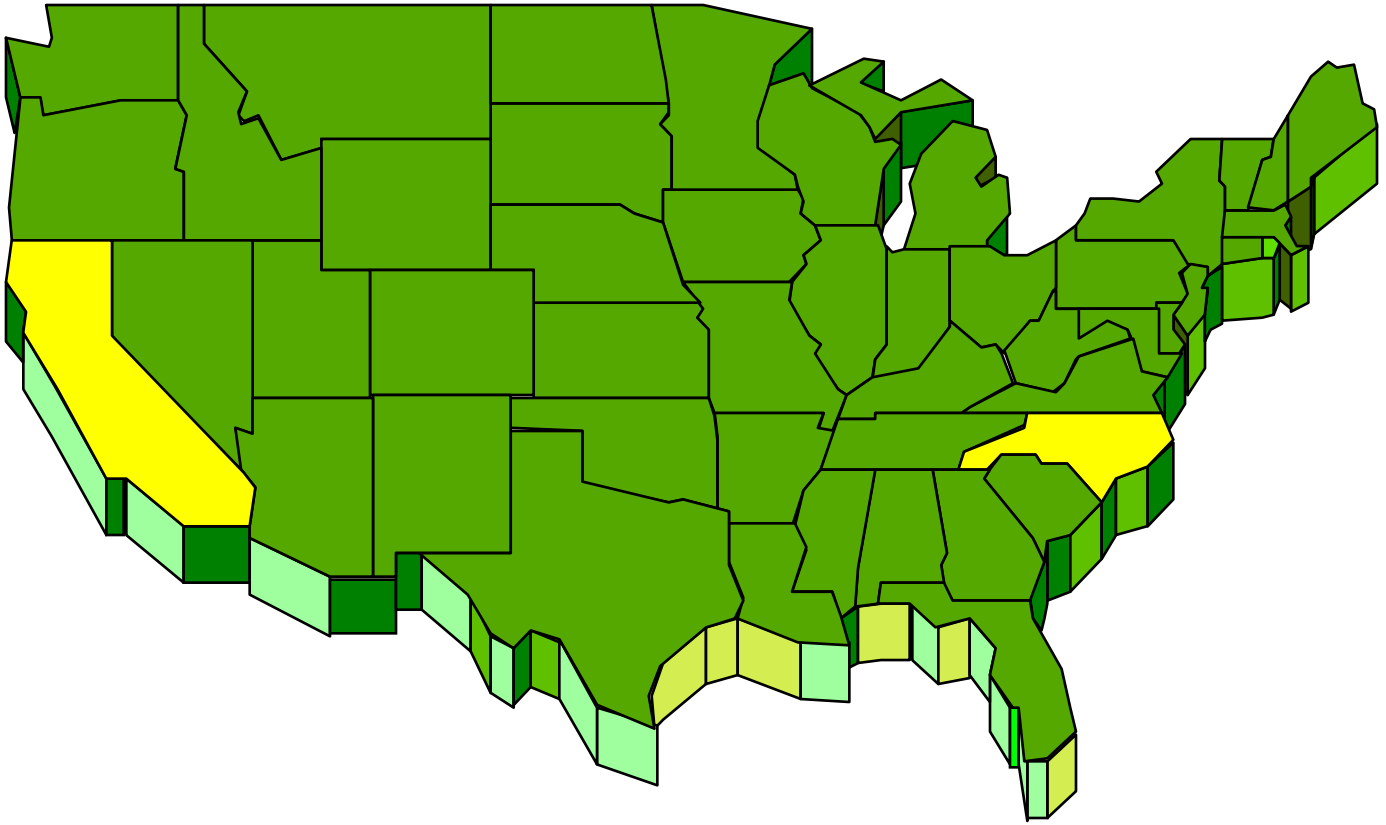
…but there are a lot of questions that I want to answer



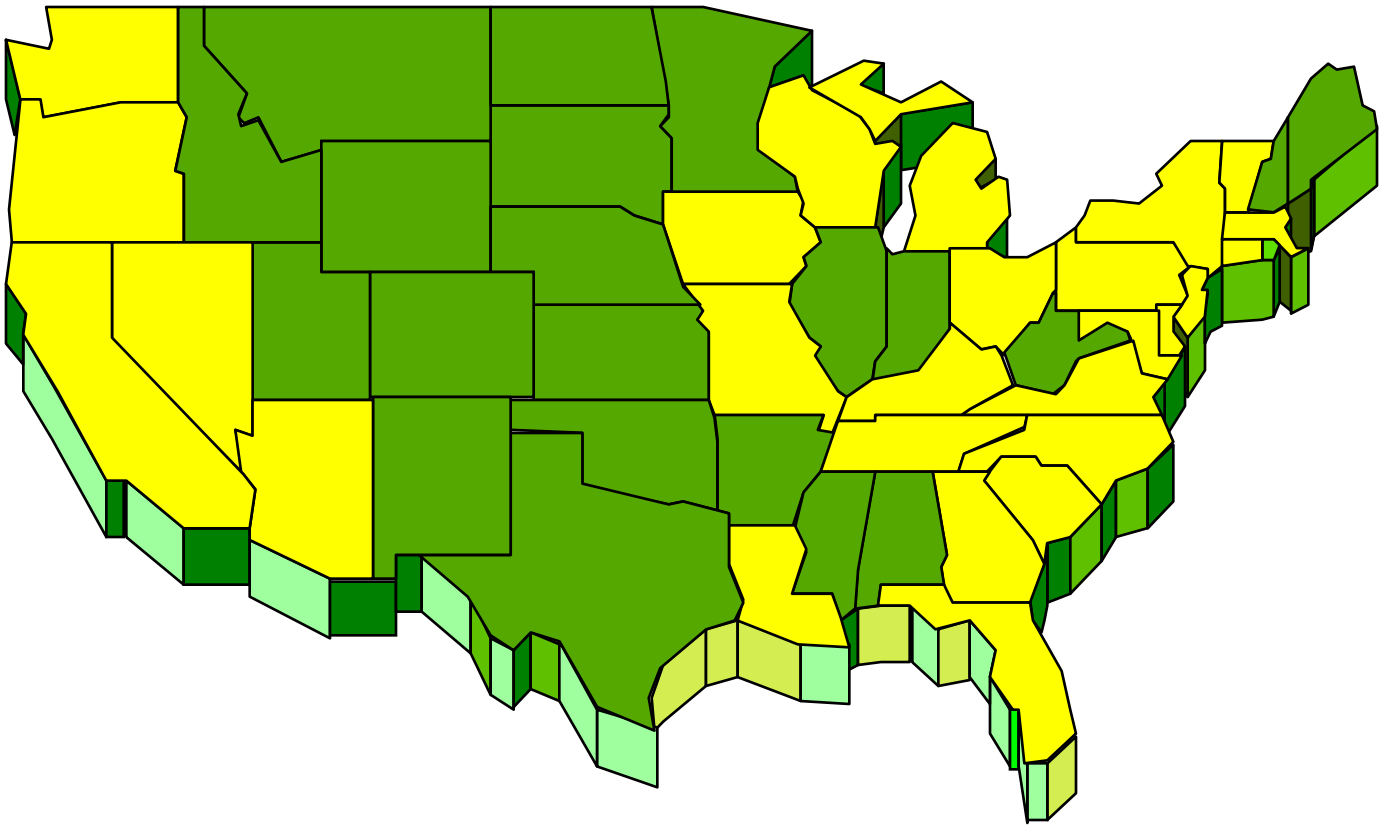
Hemolytic anemia, thrombocytopenia, hyperglobulinemia, icterus, fever, splenomegaly, and lymphadenopathy

How many dogs have it?

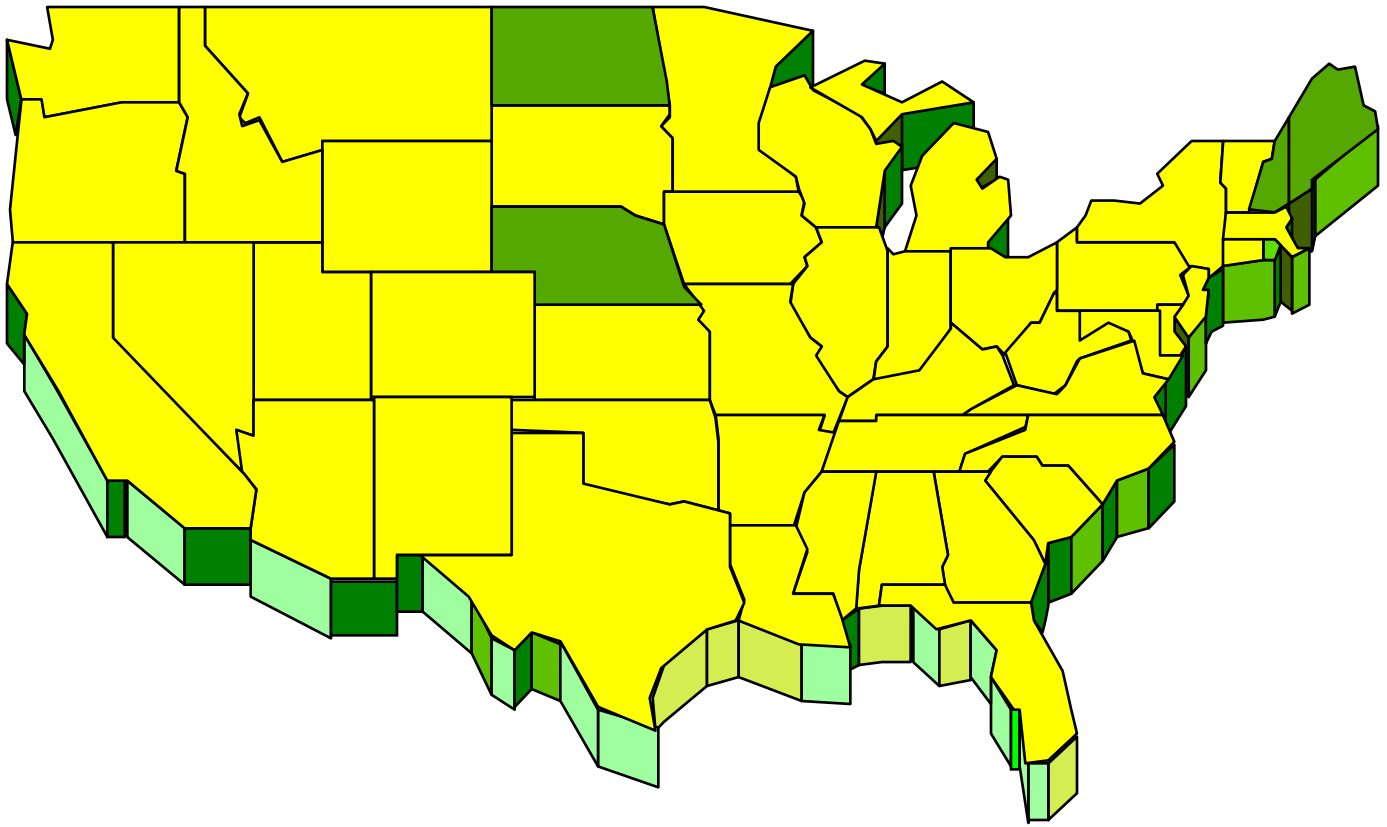
How extensive is the infection: 1995



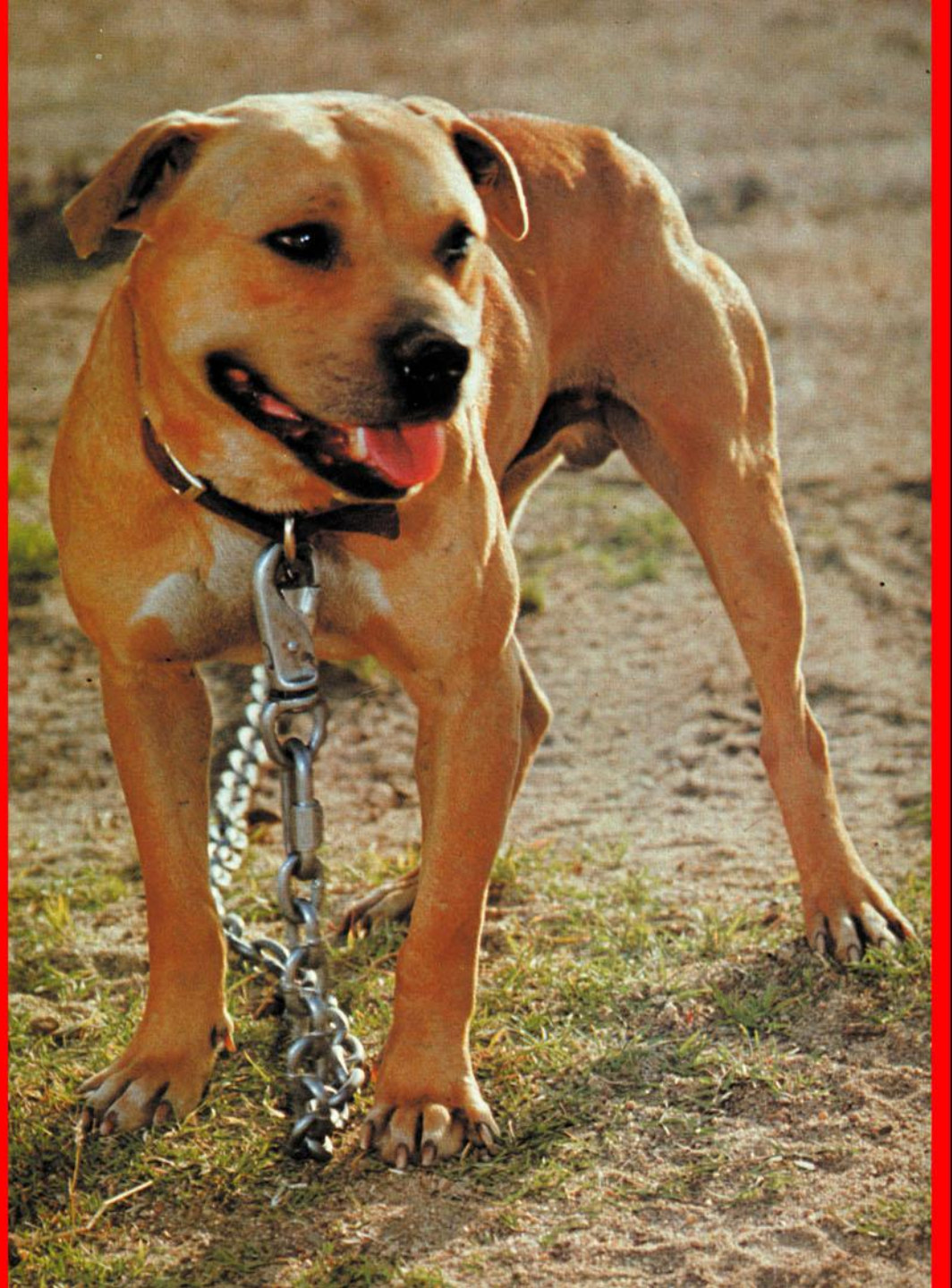
How extensive is the infection: 2003



How extensive is the infection: 2012



Typical living conditions of the kennel dogs



How is it transmitted?

- *Hypothesis: Babesia gibsoni* infections in the US are transmitted directly via dog bites and are not vector-transmitted
- APBT owners were not willing to give out information about previous bite wounds

Results

	Babesia gibsoni	*Babesia canis vogeli*	Other piroplasm
Total	**131**	**11**	**3**
APBT*	**121**	**0**	**1**
Greyhound	**0**	**8**	**0**
Other Breed	**10**	**3**	**2**

* APBT: American Pit Bull Terrier

Approach

- 15 *B. gibsoni* infected dogs that were not APBTs were identified
  - 3 excluded due to lack of known history
- 12 *Ehrlichia ewingii* infected dogs were used as a control group representing dogs with a tick-transmitted disease

	Dog bite	APBT* bite	Ticks	Housed with infected dog
Babesia gibsoni	**9/12**	**9/12**	**2/12**	**4/12**
Ehrlichia ewingii	**3/12**	**0/12**	**5/12**	**2/12**

* APBT: American Pit Bull Terrier

Results/Conclusions

- *B. gibsoni* infected dogs were more likely to have had a recent dog bite than *E. ewingii* infected dogs (Odds ratio 9.0; 95% CI 1.07 to 96.34; p = 0.014)
- The association was stronger ($p < 0.0001$) when the dog inflicting the bite was an APBT
- The association remained ($p = 0.045$) when each household with multiple infected dogs was counted as a single case

Now What?

- Developed test
- Identified epidemic
- Identified risk factors for infection
- THERE IS NO EFFECTIVE TREATMENT FOR *Babesia gibsoni*!

Treatment

- Treatments reduce morbidity and mortality but cannot clear the infections
  - Imidocarb, diminazene, trypan blue, phenamidine, doxycycline, clindamycin….
- Treated dogs are carriers and are at risk for recurrence of signs and can transmit infections to other dogs

Atovaquone and Azithromycin

- Atovaquone is a hydroxy-1,4-naphthoquinone: analog of ubiquinone and interferes with electron transport
- Azithromycin is an azalide antibiotic and interferes with protein synthesis
- Both drugs have anti-*Babesia* activity
- Together have been effective against other *Babesia* spp.

(Hughes 1995, Wittner 1996, Pudney 1997, Gray 1999, Krause 2000)

Specific Aim

- To determine whether or not an atovaquone and azithromycin drug combination would decrease *Babesia gibsoni* parasitemia below the limit of detection

Approach

- Double-blind placebo-controlled
  - 11 treatment
  - 11 placebo
- Could detect 60% difference between groups (based on pilot study)
- 3 post-treatment PCR tests
- CBC, biochemical profiles

Results

Dog	Group	Day 0	Day 60	Day 90	Day 120
1	Treatment	+	-	-	-
2	Treatment	+	-	-	-
3	Treatment	+	-	-	ND
4	Treatment	+	-	-	-
5	Treatment	+	-	-	-
6	Treatment	+	+	+	ND
7	Treatment	+	-	-	-
8	Treatment	+	-	-	-
9	Treatment	+	-	-	-
10	Treatment	+	-	-	-
11	Treatment	+	-	+	ND
12	Placebo	+	+	+	+
13	Placebo	+	+	ND	ND
14	Placebo	+	+	+	-
15	Placebo	+	+	+	+
16	Placebo	+	+	+	ND
17	Placebo	+	-	+	+
18	Placebo	+	+	+	+
19	Placebo	+	-	+	+
20	Placebo	+	+	+	+
21	Placebo	+	+	-	+
22	Placebo	+	+	+	+

+, positive PCR test
-, negative PCR test
ND, not determined

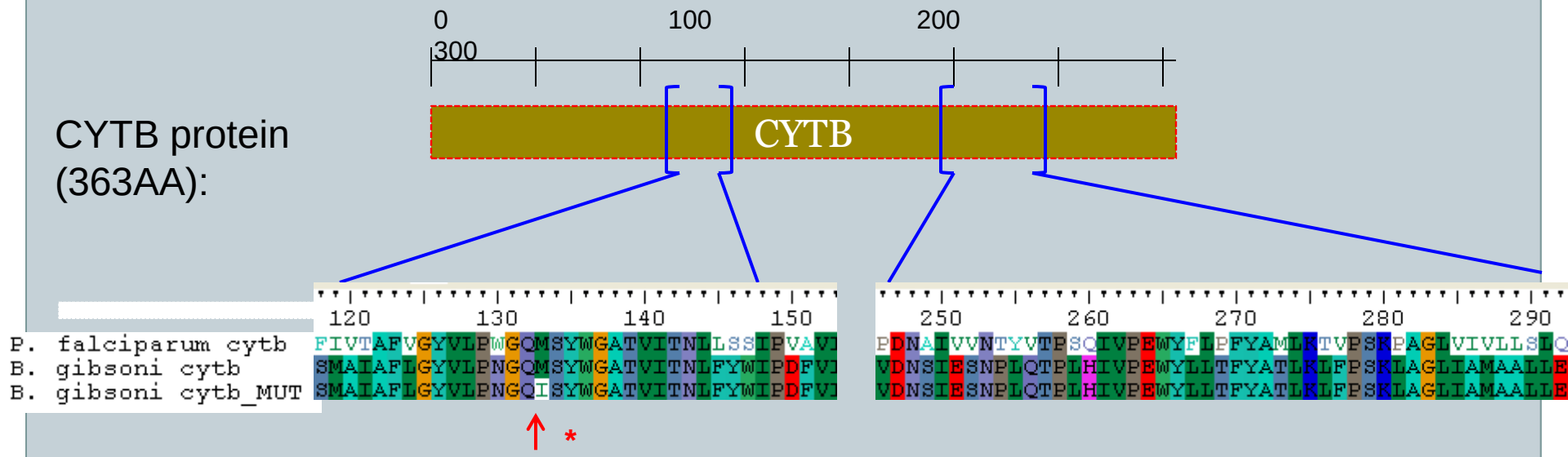
Results/Conclusions

- There was a significant difference between the treatment and placebo groups (p= 0.0001)
- No side-effects were reported in either the treatment or placebo treated dogs
- Only treatment reported to eliminate detectable *B. gibsoni* parasitemia

(Birkenheuer et. al., J Vet Internal Med, 2004)

Mechanism Atovaquone Resistance?

CYTB protein (363AA):



Am. J. Trop. Med. Hyg., 74(4), 2006, pp. 593–597

SHORT REPORT: CLONING OF THE *BABESIA GIBSONI CYTOCHROME B* GENE AND ISOLATION OF THREE SINGLE NUCLEOTIDE POLYMORPHISMS FROM PARASITES PRESENT AFTER ATOVAQUONE TREATMENT

AYA MATSUU,* KAYOKO MIYAMOTO, HIROMI IKADAI, SHOZO OKANO, AND SEIICHI HIGUCHI

Department of Small Animal Internal Medicine 1, Department of Veterinary Parasitology, and Department of Small Animal Surgery 3, School of Veterinary Medicine and Animal Sciences, Kitasato University, Aomori, Japan

J Vet Intern Med 2009;23:493–498

Possible Emergence of Drug-Resistant Variants of *Babesia gibsoni* in Clinical Cases Treated with Atovaquone and Azithromycin

M. Sakuma, A. Setoguchi, and Y. Endo

- 5/8 dogs relapsed after treatment
- ALL of the relapsed dogs had M121I mutation AFTER treatment

Molecular Epidemiological Survey of the *Babesia gibsoni cytochrome b* Gene in Western Japan

Masato SAKUMA[1)], Kenta FUKUDA[2)], Katsuyoshi TAKAYAMA[2)], Yukuharu KOBAYASHI[2)], Takako SHIMOKAWA MIYAMA[3)], Asuka SETOGUCHI[1)] and Yasuyuki ENDO[1)]*

- Sequenced cytochrome B gene from 92 dogs with B. gibsoni
- NO previous treatment (not clear how this was confirmed)
- 3/92 dogs had M121I mutations

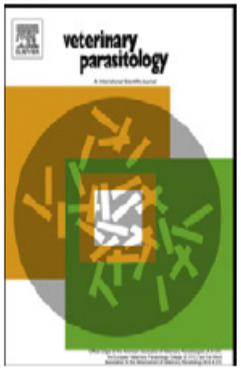
How is this possible if 5/8 dogs fail treatment?

Veterinary Parasitology 185 (2012) 145–150

Contents lists available at SciVerse ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar



Development of in vitro atovaquone-resistant *Babesia gibsoni* with a single-nucleotide polymorphism in *cytb*

Aiko Iguchi[a], Aya Matsuu[a,*], Hiromi Ikadai[b], Md. Hasanuzzaman Talukder[c], Yoshiaki Hikasa[a]

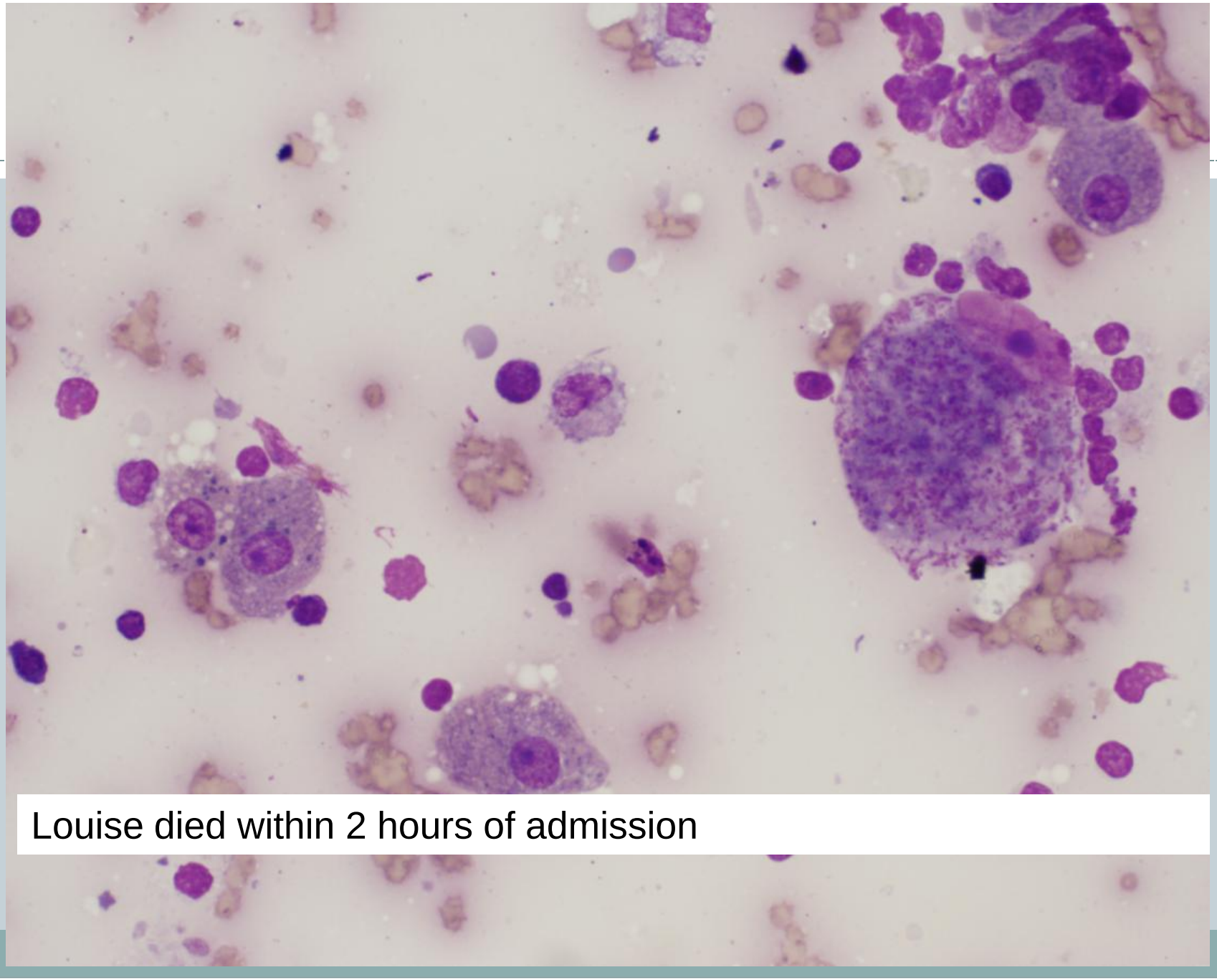
In vitro atv-resistant strain

- They “induced” atv-resistance in vitro
- From wild-type and “cloned” strains
- M121 mutation was selected for NOT induced

Why the differences between USA and Asia?

August 10, 2003

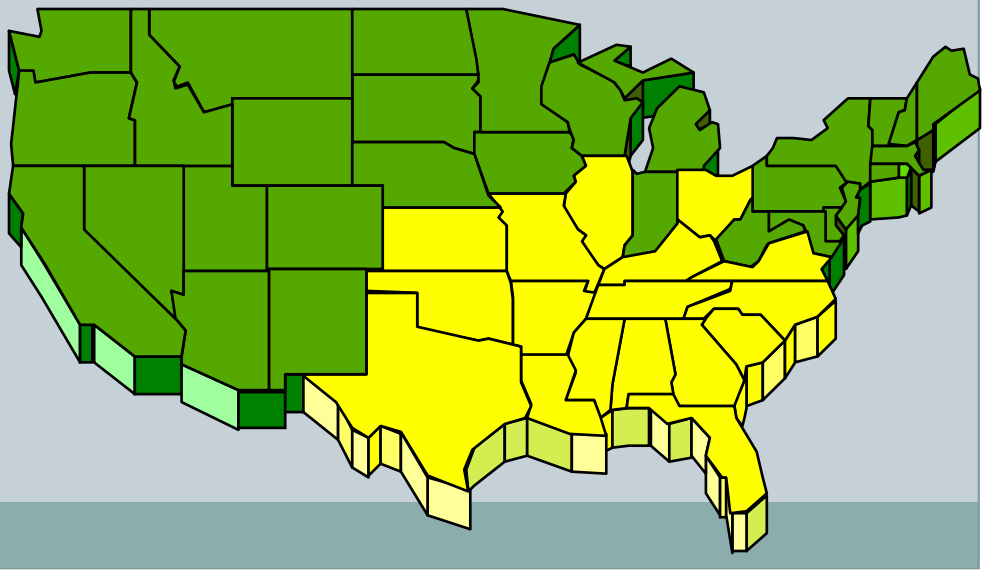
- "Louise"
- 8 yr FS DSH
- **History-full** - Brought in by the pet sitter so some of recent history is unknown. Owners are out of town and she has left a message for them. Inside and outside kitty in a multi-cat household. Unsure of systems review but did see the cat eating on Thursday. Last night was laying around and somewhat unresponsive. Limp and wobbly this morning. (Drs. Birkenheuer & Concannon)

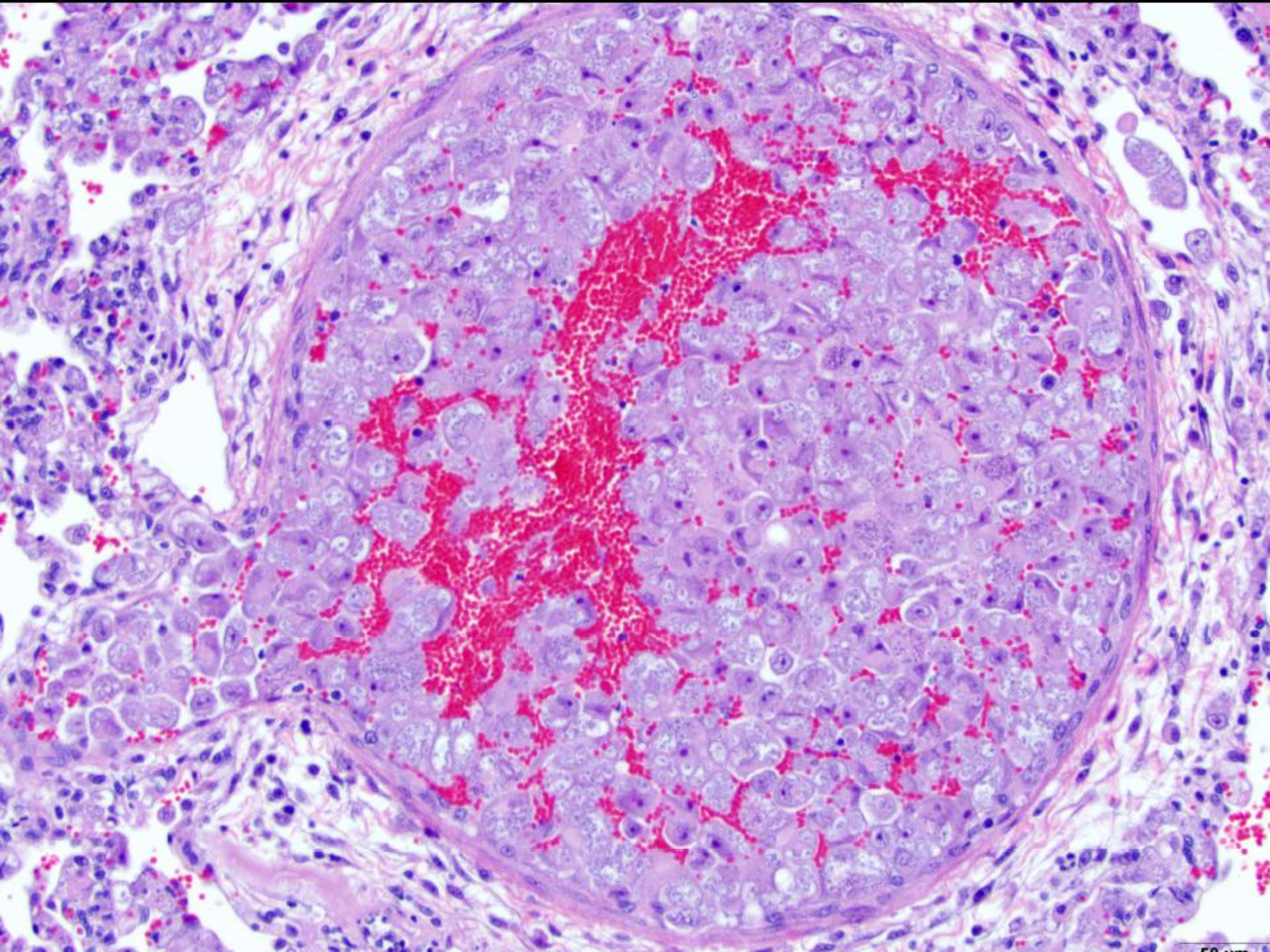


Louise died within 2 hours of admission

Cytauxzoon felis

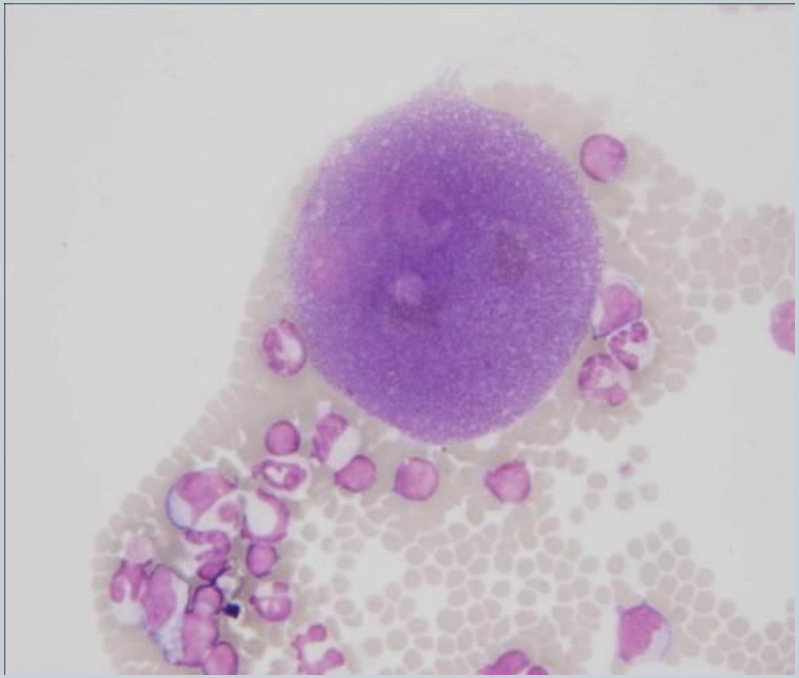
- Tick-borne protozoan parasite that can fatally infect domestic cats and is harbored in bobcats
- Present in southeastern and south-central United States
- Discovered in 1976
- Only detected in carolinas in past decade
- Related to *Babesia, Theileria, and Plasmodium* species





Survival Post Infection: Historical Perspective

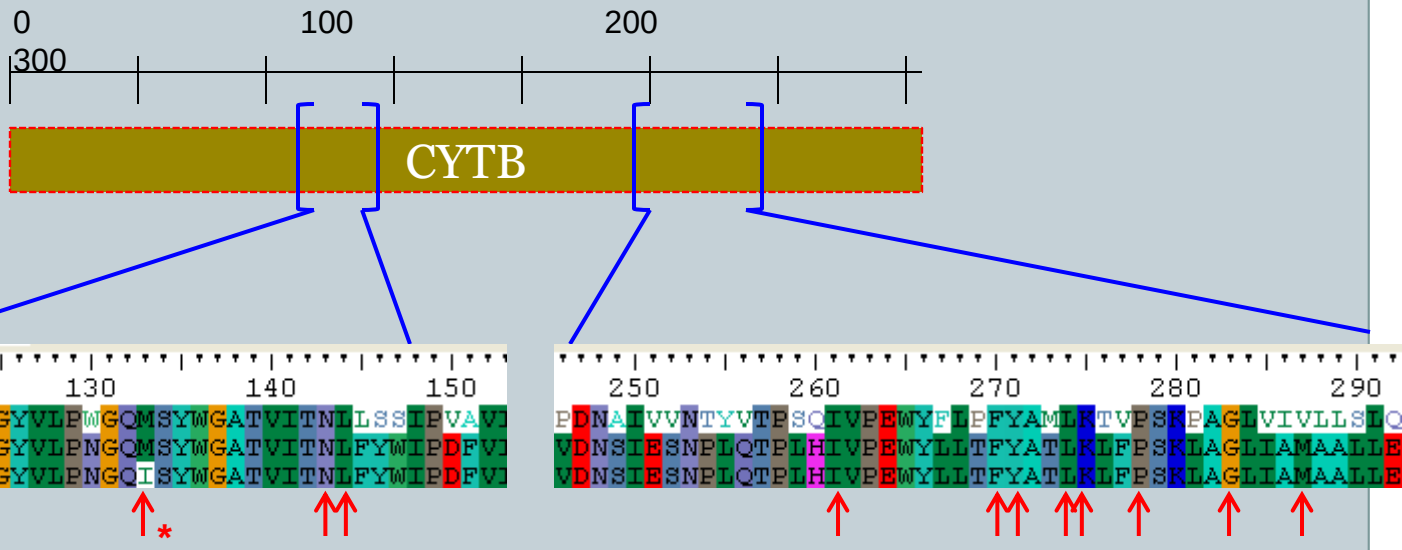
- **Initial discovery: believed to be 100% fatal**
- **Plum Island studies: 500 infected, 1 survived**
- **Current treatment with atovaquone and azithromycin: 60% survival**



Atovaquone Resistance

Atovaquone=anti-malarial drug that targets cytochrome *b* in the mitochondrial genome:

CYTB protein (363AA):



Sick of hunting for one gene at a time

Features	*P. falciparum*†	*T. parva*†	*B. bovis*†	*B. gibsoni*	
				Genemark	Glimmer
Genome Size (Mbp)	22.8	8.3	8.2	7.5	7.5
G+C composition (%)	19.4	34.1	41.8	44	44
Protein Coding Genes	5,268	4,035	3,671	3,471	3,458
Average Protein Length	761	469	505	520	472
% Genes With Introns	53.9	73.6	61.5	68.65	61.1
Protein coding (%)	52.6	68.4	70.2	71.3	65

Features	*P. falciparum*[2]	*B. bovis*[2]	*T. parva*[2]	*C. felis*	
				GeneMark	Glimmer
Genome Size (Mbp)	22.8	8.2	8.3	9.1	9.1
G+C Composition (%)	19.4	41.8	34.1	31.8	31.8
Protein Coding Genes	5,268	3,671	4,035	4,314	4,373
Average Protein (aa)	761	505	469	466.32	408.52
% Genes With Introns	53.9	61.5	73.6	68.65	61.1
Protein Coding (%)	52.6	70.2	68.4	66.2	68.8

Hypothesis

- *C. felis cytb* genotypes are associated with response (i.e. survival) to atovaquone and azithromycin treatment
- Cats that died were infected with *C. felis* strains that have missense mutations within or near the atovaquone binding site of the *cytb* gene.

Methods: Cats from Treatment Study

J Vet Intern Med 2011;25:55–60

Efficacy of Atovaquone and Azithromycin or Imidocarb Dipropionate in Cats with Acute Cytauxzoonosis

L.A. Cohn, A.J. Birkenheuer, J.D. Brunker, E.R. Ratcliff, and A.W. Craig

Background: Imidocarb or a combination of atovaquone and azithromycin (A&A) has been suggested for treatment of cats with cytauxzoonosis, but neither has been prospectively evaluated for efficacy.

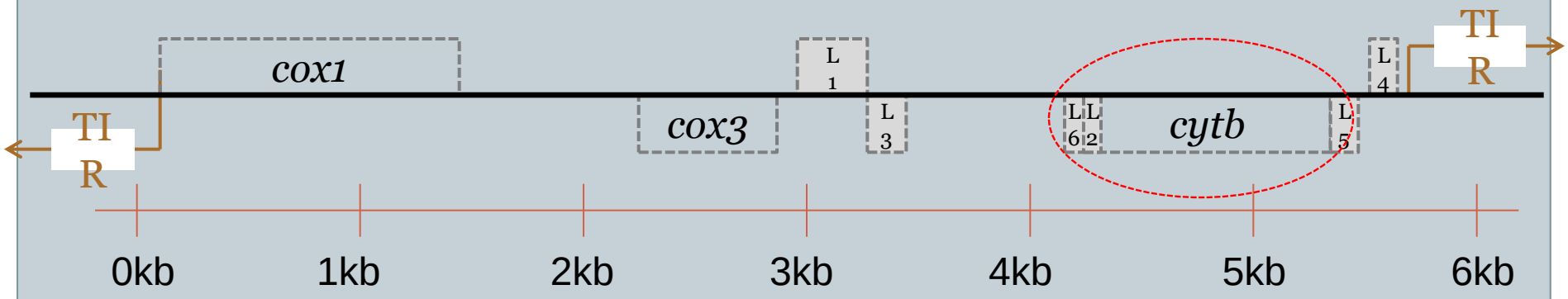
Hypothesis/Objectives: That survival to hospital discharge is improved by treatment with A&A as compared with imidocarb.

45 cats with *C. felis* infection that were **treated with atovaquone and azithromycin**

- **28/45 (62%) survived post treatment**

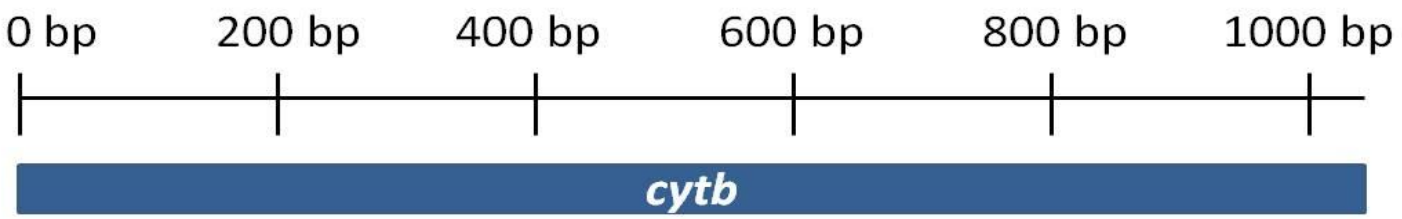
Great idea! But…

C. felis cytb gene wasn't in our genome!



PCR Assay

Cytb gene (1092 b

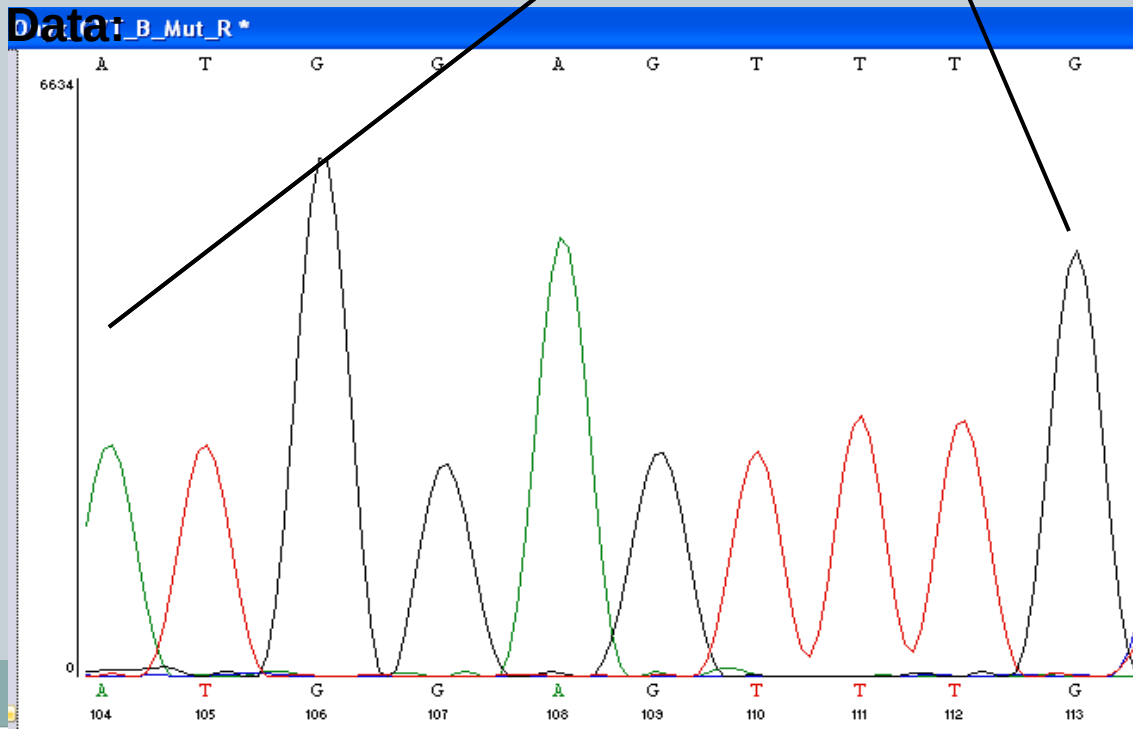


Sequence Analysis

Text File:

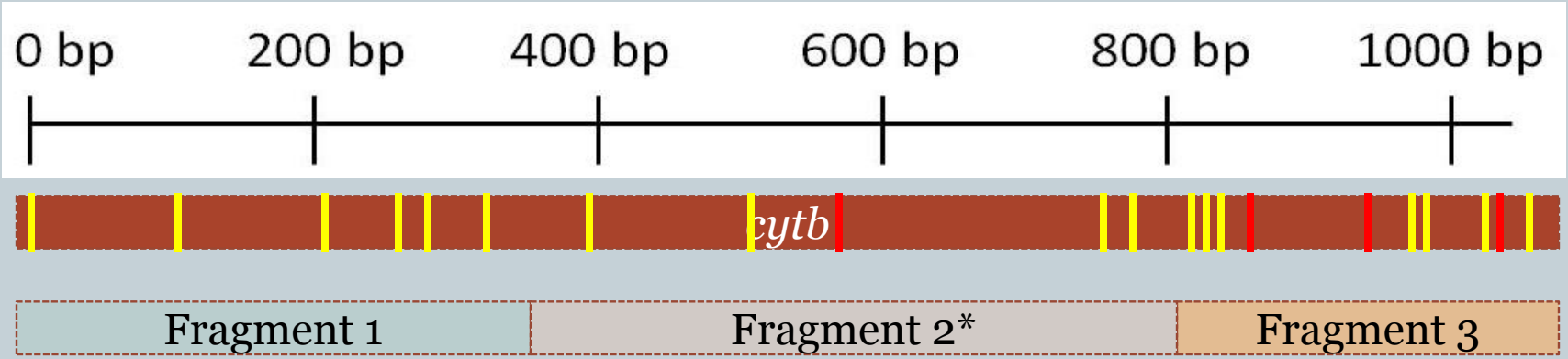
```
                    '|'''''|'''''|'''''|'''''|'''''|'
                    100         110         120
Onyx cytb MUT  R    ATGCAATGGAGTTTGCAATGGAT
```

Chromatogram Data:



RESULTS

High Diversity Present in *cytb* Gene



- 21 mutations: all substitutions
- 22 unique genotypes
- 4 mutations caused AA changes

Cytb Genotypes

Amino Acid Position	1	39	69	85	92	108	133	169	193	250	257	276	279	286	288	316	327	328	344	345	357	
Nucleotide Position	3	115	207	255	274	324	399	507	578	750	771	826	835	858	862	947	981	984	1032	1035	1071	# Samples
GENOTYPE																						
1	G	T	C	T	C	T	A	T	A	A	T	T	C	G	A	T	C	T	T	A	T	8
2	G	C	C	C	C	T	A	T	A	G	T	T	T	G	A	T	C	T	C	A	T	3
3	G	T	C	T	C	T	A	T	A	A	T	T	C	G	A	C	C	T	T	A	T	4
4	G	C	C	C	C	T	A	T	A	G	C	C	T	G	A	T	C	C	C	A	T	3
5	G	C	C	C	C	C	A	T	A	G	T	T	T	G	A	T	C	T	C	A	T	4
6	G	C	C	C	C	T	A	T	A	G	Y	Y	T	G	A	T	C	Y	C	A	T	2
7	G	Y	C	Y	C	Y	A	T	A	R	T	T	Y	G	A	T	C	T	Y	A	T	3
8	G	Y	C	Y	Y	T	A	T	A	R	T	T	C	G	A	T	C	T	Y	A	T	2
9	G	Y	C	Y	Y	Y	A	T	A	G	T	T	T	G	A	T	C	T	C	A	T	1
10	G	T	C	T	C	T	G	T	A	A	T	T	C	G	A	T	C	T	T	A	C	2
11	G	C	C	C	T	T	A	T	A	G	T	T	T	G	A	T	C	T	C	A	T	2
12	G	C	C	C	C	C	A	C	A	G	T	T	T	G	A	T	C	T	C	A	T	1
13	G	C	C	C	C	T	A	T	A	G	T	T	T	G	A	T	T	T	C	G	T	1
14	G	C	C	C	C	T	A	T	A	G	T	T	T	G	R	T	T	T	C	A	T	1
15	G	C	C	C	C	T	A	T	A	G	T	T	Y	G	A	T	C	Y	C	A	T	1
16	G	C	C	T	C	T	A	T	A	G	T	T	T	G	A	T	C	T	C	A	T	1
17	G	T	C	T	C	T	A	T	A	A	T	T	C	A	A	T	C	T	T	A	T	1
18	G	T	C	T	Y	T	A	T	A	A	T	T	C	G	A	T	C	T	T	A	T	1
19	G	Y	C	Y	C	T	A	T	A	R	T	T	C	G	A	T	C	T	Y	A	T	1
20	G	Y	C	Y	C	T	A	T	R	R	Y	Y	Y	G	A	T	C	Y	Y	A	T	1
21	G	Y	C	Y	Y	Y	A	Y	A	G	T	T	T	G	A	T	C	T	C	A	T	1
22	R	Y	Y	Y	C	Y	A	T	A	R	T	T	Y	G	A	T	C	T	Y	A	T	1

Survival is associated with WT *cytb* genotype

	Survived	Died	Total
WT	8	0	8
Non-WT	20	17	37
Total	28	17	45

p=0.017 Fisher's Exact Test, two-tailed p-value

…is this really associated with atovaquone treatment?

New aim introduced to study…

J Vet Intern Med 2011;25:55–60

Efficacy of Atovaquone and Azithromycin or Imidocarb Dipropionate in Cats with Acute Cytauxzoonosis

L.A. Cohn, A.J. Birkenheuer, J.D. Brunker, E.R. Ratcliff, and A.W. Craig

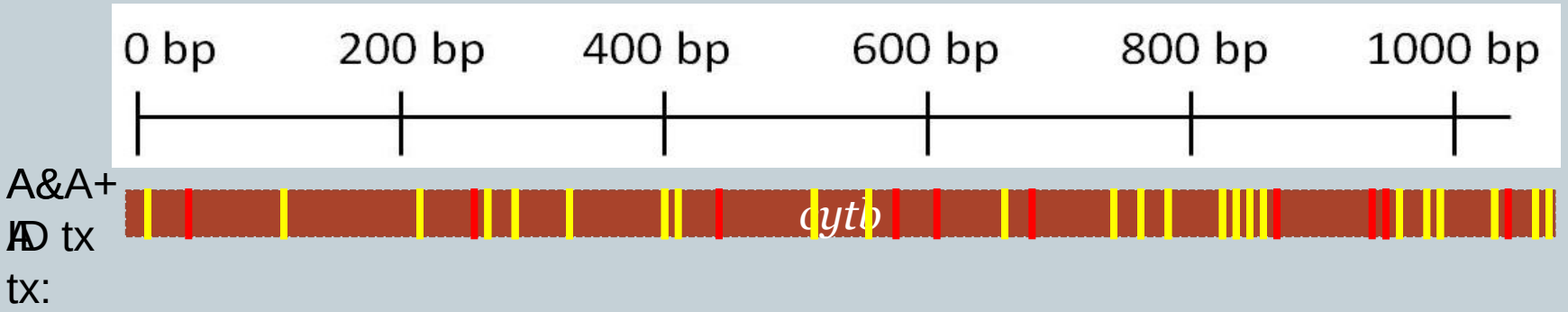
Background: Imidocarb or a combination of atovaquone and azithromycin (A&A) has been suggested for treatment of cats with cytauxzoonosis, but neither has been prospectively evaluated for efficacy.

Hypothesis/Objectives: That survival to hospital discharge is improved by treatment with A&A as compared with imidocarb.

24 additional cats with *C. felis* infection that were treated with **imidocarb dipropionate**

- **7/24 (29%) survived post treatment**

Imidocarb Dipropionate Treatment Group



- 14 mutations (all substitutions)
- 8 more unique genotypes
- 6 mutations caused AA changes
- All samples: 35 mutations, 30 unique genotypes
- WT genotype represented in highest frequency (n=5); **but is there an association with survival?**

WT *cytb* genotype and survival appear to be associated with atovaquone/azithromycin treatment

Atovaquone and Azithromycin

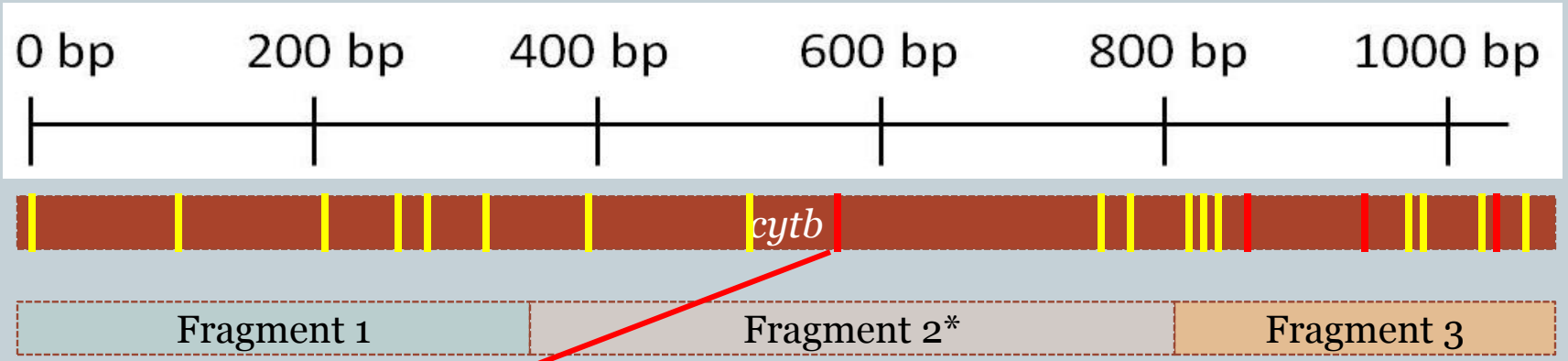
	Survived	Died	Total
WT	8	0	8
Non-WT	20	17	37
Total	28	17	45
p=0.017			

Imidocarb Dipropionate

	Survived	Died	Total
WT	2	3	5
Non-WT	5	14	19
Total	7	17	24
p=0.608			

- First part of hypothesis appears to be true: there is an association between *C. felis cytb* genotype and survival with atovaquone/azithromycin treatment
- But what about the second part of the hypothesis?

Hypothesis: Cats that died were infected with *C. felis* strains that **have missense mutations within or near the atovaquone binding site** of the *cytb* gene.



1 genotype with missense mutation in atovaquone binding site: cat *did* die

BUT…

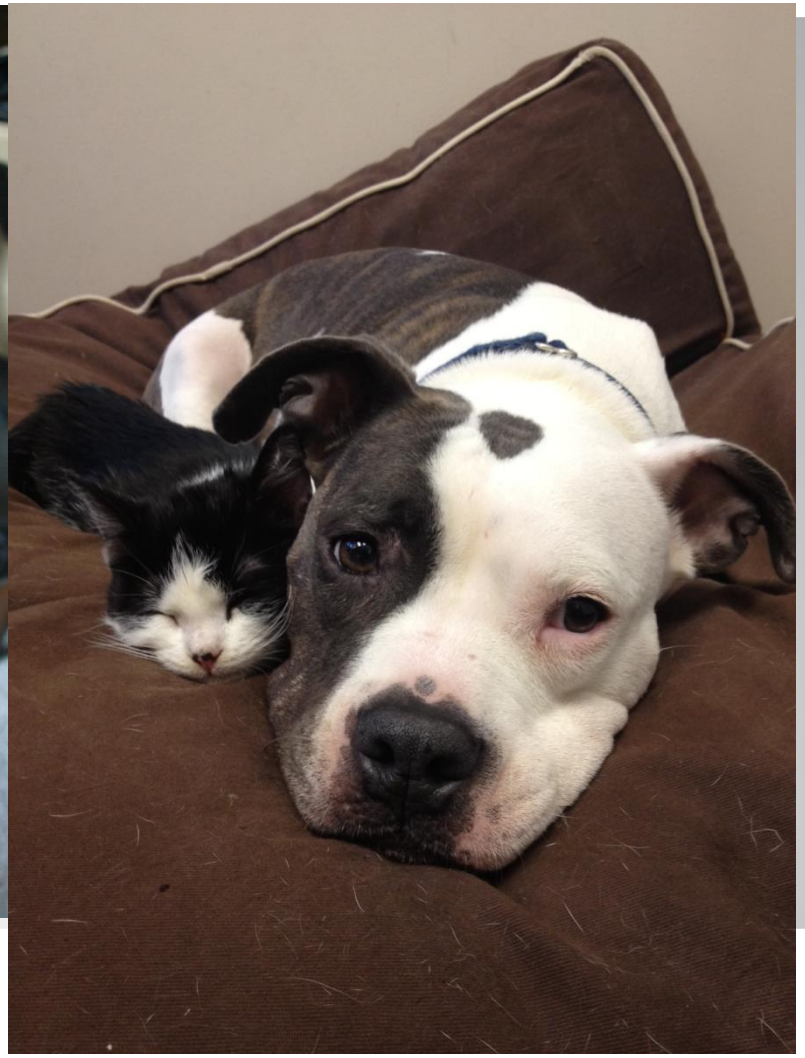
16 other cats in this treatment group died without missense mutations

Missense mutations are not responsible for lack of response to atovaquone treatment!

How could the WT *cytb* genotype have an effect?

1. Transcription and translation
2. Could be linked to mutations in cytochrome oxidase or rRNA genes

Questions?



Questions?